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Evolutionary history of the ABCB2 genomic region in teleosts ☆

Y. Palti[a,*], M.F. Rodriguez[a,1], S.A. Gahr[a], J.D. Hansen[b]

[a] *National Center for Cool and Cold Water Aquaculture USDA/ARS, 11861 Leetown Road, Kearneysville, WV 25430, USA*
[b] *Western Fisheries Research Center—USGS Biological Resources Division, Department of Pathobiology, University of Washington, Seattle, WA 98115, USA*

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Abstract

Gene duplication, silencing and translocation have all been implicated in shaping the unique genomic architecture of the teleost MH regions. Previously, we demonstrated that trout possess five unlinked regions encoding MH genes. One of these regions harbors ABCB2 which in all other vertebrate classes is found in the MHC class II region. In this study, we sequenced a BAC contig for the trout ABCB2 region. Analysis of this region revealed the presence of genes homologous to those located in the human class II (ABCB2, BRD2, ψDAA), extended class II (RGL2, PHF1, SYGP1) and class III (PBX2, Notch-L) regions. The organization and syntenic relationships of this region were then compared to similar regions in humans, Tetraodon and zebrafish to learn more about the evolutionary history of this region. Our analysis indicates that this region was generated during the teleost-specific duplication event while also providing insight about potential MH paralogous regions in teleosts.
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1. Introduction

Presentation of intracellularly derived peptides by MHC class Ia molecules is an important component of cell-mediated immunity against viral pathogens. Class Ia molecules are sequestered in the endoplasmic reticulum (ER) until they are loaded with appropriate peptide antigen and released to the cell surface for surveillance by CD8+ cytotoxic T lymphocytes (CTLs). Endogenous proteins resulting from intracellular pathogens are processed into small antigenic peptides (8–11 mers) by the immunoproteasome and then transported into the lumen of the ER from the cytosol via the transporters associated with antigen presentation, ABCB2 and ABCB3 (TAP1 and 2) [1]. In mammals, the class I peptide-loading complex consists of a single ABCB2/3 heterodimer, four tapasin (TAPBP) molecules and four class I-β2m molecules [2], where TAPBP serves as a bridge between the peptide transporters and the class I/b2M complex. The genes encoding the MHC class Ia pathway, together with the MHC class Ib, II and III genes are physically linked forming the major histocompatibility complex in mammals. Similar genomic organizations are observed in all vertebrates studied to date with the exception of bony fish. Bony fish

☆ The genomic contig described in this paper has been deposited in GenBank under accession number DQ246664.

* Corresponding author. Tel.: +1 304 724 8340; fax: +1 304 725 0351.
E-mail address: yniv.palti@ars.usda.gov (Y. Palti).
[1] Current address: Monsanto Co., 800 N. Lindbergh Building, U2C, Street Louis, MO 63167, USA.

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possess MH "regions" [3,4] as the class Ia, Ib and class II genes reside on separate chromosomes. This phenomenon was first described in zebrafish [5] and later in other teleosts including medaka [6], fugu [7] and rainbow trout [4,8].

In recent years, much of the effort in teleost MH genomics has been directed toward comparative studies of the genomic structure of the teleost MHC class I region which identified a high degree of conserved synteny among the teleosts class I region [4,9–12]. Interestingly, most of the genes that are involved directly in the class Ia antigen presentation pathway including PSMB8, PSMB9, PSMB9-like, PSMB10, ABCB3 and TAPBP are linked in the fish MHC class I genomic region forming the class I "core" region, with the exception of ABCB2. From the structural viewpoint, however, there is nothing in common among the MHC class Ia, PSMBs and ABCB2 and 3 molecules. Therefore, the underlining assumption of most comparative studies involving the MHC suggests that the clustering of these genes is the result of selection pressure requiring co-evolution and co-regulation of the MHC-related genes (recently reviewed by [13]).

Previously we demonstrated that the rainbow trout MH class I core region is duplicated, that ABCB2 maps to a separate linkage group (LG) and chromosome and that the class II region is localized to yet another LG and chromosomal region [4]. The extent of the class I duplication event was recently formalized via BAC sequencing of the core regions for the class IA and B regions [12]. More recently we determined that TAPBP is part of the trout class Ia core region and that the trout tapasin-related gene (*Onmy*-TAPBP-R.1) and ABCB2 are co-localized on the short arm of chromosome 2, while *Omny*-TAPBP-R.2 is located on chromosome 3 representing a fifth MH chromosomal region in rainbow trout [14].

Based upon prior studies of the trout MH regions [4,12], we analyzed the ABCB2 locus in trout to determine if genes encoded within this region could lend new insight as to the unique MH architectural arrangements observed in bony fish. In this report, we present the sequence of a 320 kbp chromosomal region (Chr. 2) containing the rainbow trout ABCB2 gene and an initial genomic characterization of the expressed genes in this region to identify conserved synteny with other vertebrates. The identification of BRD, PBX and NOTCH-like genes along with ABCB2 within this region suggests that this region is the likely remnants of the ancestral MHC synteny. Therefore this is the first report describing the genomic organization of this "lost" MH region in teleosts enabling future studies exploring the possible functional and evolutionary significance of conserved synteny in this region and the potential roles of the encoded genes for fish health.

2. Materials and methods

2.1. BAC library screening and DNA fingerprinting analysis

The Swanson 10X rainbow trout BAC library which was derived from a doubled-haploid homozygous line, was previously screened using radiolabeled probes hybridization to identify clones that harbor the ABCB2 gene [15]. DNA was isolated from 15 positive BACs (Qiagen Corp., Valencia, CA) and digested with HindIII (Promega, Madison, WI). The generated banding patterns were analyzed using Image/FPC software [16] to assemble contigs of overlapping BACs [15].

2.2. BAC sequencing and gene annotation

Three BACs that represent a contig of approximately 320 kbp were processed using the Qiagen Large Construct kit for the construction of BAC DNA shotgun libraries. BAC DNA was sheared into 1- to 3-kbp fragments, subcloned into pBSK^{+}, sequenced to 9× coverage, and assembled using the PHRED-PHRAP-CONSED software package [17,18]. Only Phred values of >20 were used for the assembly. Primers for gap filling were derived from the sequence of sub-clones that overlapped sequence gaps. Sequence assembly was confirmed by comparing the actual HindIII restriction digestion pattern of each BAC and the in silico digestion pattern predicted by the PHRAP/CONSED virtual digestion tool. The assembled sequence contig from the three BAC clones was annotated using GeneScan [19] (http://genes.mit.edu/GENSCAN.html) including manual adjustments. Full-length sequences of EST cDNA clones displaying high identity ($\geqslant 98\%$) to the ORFs (GenBank accession numbers DR782904) were used to refine intron/exon boundaries using Sequencher (Gene Codes Corporation, Ann Arbor, MI). Gene names, abbreviations and locations in the human genome were inferred from Gene Cards (http://bioinfo.weizmann.ac.il/cards/index.shtml). Gene locations in the *Tetraodon nigroviridis* and zebrafish genomes were inferred by predicted peptides sequence similarity

(http://www.ensembl.org/Tetraodon_nigroviridis/ and http://www.ensembl.org/Danio_rerio/, respectively).

2.3. Gene expression analysis

Rainbow trout ESTs with high sequence similarity to the putative genes located within the 320 kbp genomic contig were identified using BLASTN [20] (http://www.ncbi.nlm.nih.gov/BLAST/). A primer walking method was used to obtain the complete sequence of the EST cDNA clones (DR782904, DQ139863, DQ143175-9). Reverse transcriptase (RT) PCR was conducted to verify the expression of ORFs that were absent from the trout EST gene index [21] and from a putative BRD2 ortholog that was present in the locus/EST gene index to serve as a positive control. Total RNA from indicated tissues was isolated from a healthy adult female rainbow trout (except testis from a healthy full-sib male) using TRI-Reagent (Sigma-Aldrich Corp., St. Louis, MO). Reverse transcription reactions were performed using 2 μg of total RNA, as described by [22]. Tissue-specific expression of selected genes was determined by gene-specific amplification (RT-PCR) including β-actin as the housekeeping gene. The primer sequences and expected genomic and cDNA amplicon size for each of the four putative genes are presented in Table 1. The PCR reactions (10 μl total volume) included 25 ng cDNA, 0.5 μM of each primer, 200 μM dNTPs, 1 × buffer (Qiagen Hotstar Taq 10 × PCR buffer), and 0.5 units of Hotstar Taq polymerase (Qiagen, Valencia, CA). Amplifications were conducted as follows: initial denaturation at 94 °C for 10 min, 30 cycles consisting of 94 °C for 45 s, 58 °C for 45 s, 72 °C extension for 45 s; followed by a final extension of 72 °C for 10 min. PCR products were separated on 2% agarose gel and stained with EtBr for visualization with the Typhoon imaging system (Model 9210, Amersham Biosciences, Piscataway, NJ). PCR products were verified both by fragment size and direct sequencing.

2.4. Phylogenetic analysis and conserved synteny

Homologous genes from other species were identified for all expressed ORFs using BLASTP and tBLASTn. Amino acid sequences were aligned using ClustalW [23] (http://www.ebi.ac.uk/clustalw/) and *MEGA3* [24] (http://www.megasoftware.net/) was then used for the construction of phylogenetic trees. The phylogenetic trees were generated from the consensus of neighbor-joining algorithms (Poisson correction/exclusion of gaps) supported by 1000 repetitions of bootstrap analysis. Syntenic relationships were deduced by identifying the genome locations of the putative homologs from the phylogenetic analyses with the genome sequences of human (http://bioinfo.weizmann.ac.il/cards/index.shtml) zebrafish (http://www.ensembl.org/Danio_rerio/) and Tetraodon (http://www.ensembl.org/Tetraodon_nigroviridis/).

3. Results

3.1. Gene content of the trout ABCB2 BAC contig

In order to learn more about the history and gene content of MHC architectural arrangements in vertebrates, we isolated overlapping BACs for the

Table 1
PCR primers and the amplicon size of the cDNA and the genomic sequence used in RT-PCR expression analysis conducted for four ORFs from the rainbow trout ABCB2 region and the house keeping gene β-actin

Gene	Primer pair	cDNA size (bp)	Genomic size (bp)
BRD2	F: caaaccgatgaggactccgaagag R: aagtaggcgaagcgtagtagg	152	672
PHF1	F: gatgccccacctcttaaa R: gacacattgccgacatatcc	129	829
SYGP1	F: gactgacaagaagagacgcaagga R: tggtctggtaacgggacttga	191	441
MHC II-α (ψ)	F: aaacatcactagcgacaaag R: ttcattggagtagtactggg	229	1129
β-actin	F: cagccctccttcctcggtat R: agcaccgtgttggcgtaca	110	N/A[a]

[a]The β-actin primers are composed of exon sequences that flank a large intron, and therefore do not amplify genomic DNA [50].

ABCB2 genomic region which was previously shown to be located on a separate chromosome from the class I and II MH regions in trout. Screening of the rainbow trout Swanson 10X BAC library with the ABCB2 probe identified 15 BAC clones that formed a single contig by DNA fingerprinting analysis suggesting that the ABCB2 locus is not duplicated [15]. The complete nucleotide sequence for three overlapping BAC clones were assembled into a single contig of 319,300 bp (Fig. 1; GenBank accession number DQ246664). A total of 22 ORFs were identified within the 320 kbp contig by Genscan analysis. Eight ORFs were homologous to genes (Table 2) that are located within the human MHC class II, class III and extended class II regions [25,26] as determined by BLAST similarity profiles. Analysis of the trout EST gene index (Table 3) and results from RT-PCR (Fig. 3) indicate that all eight genes are expressed in rainbow trout.

One truncated GenScan-predicted ORF which was located near BRD2 displayed 50% amino acid identity to exon 3 of the trout class IIA chain [27] including absolute conservation of cysteine and tryptophan residues required for the Ig fold as shown in Fig. 2. Examination of genomic sequences (DQ246664) 5′ and 3′ of the DAA orphan exon were negative for additional class IIA exons. The 3′ exons and introns of this GenScan-predicted ORF had high sequence similarity to non-coding repetitive elements from other genomic regions of Chinook salmon, rainbow trout and Atlantic salmon (accession numbers AY493564, AY872256S2, AB162342, AY785950) suggesting that the remaining portion of the original DAA gene was lost via insertion and deletion events.

ABCB2 is the only gene in this region known to be directly involved in antigen processing and presentation. Rab2 (a Ras-related small GTPase encoded by RGL2) is a regulator of vesicle transport from the ER to the Golgi complex [28] and thus may affect MHC class I and class II processing. The Notch family of transmembrane receptors has been implicated in the regulation of many developmental processes and unregulated Notch activity has been reported in a variety of human cancers [29–31]. SYGP1 encodes a synaptic GTPase activating protein which is an important brain specific Ca^{2+}-dependent signaling Ras effector molecule involved in neuron synaptic signaling and regulation of spine formation in mammals [32,33]. LGN is an important regulator of spindle alignment in asymmetric cell division during development and differentiation of mammalian skin cells [34,35].

Three other expressed genes identified in this region contain DNA binding motifs and are likely to be involved in gene regulation. BRD2 is a transcriptional regulator of B cells [36] and mutations in the BRD2 promoter are the likely cause of common juvenile myoclonic epilepsy [37]. PBX2 has an important role in megakaryocytic gene expression [38] and is involved in the regulation of the proto-oncogene HOX11 [39]. Finally, the PHD finger protein (PHF) family is involved in gene regulation via protein–DNA and protein–protein interactions [40,41]. PHF1 also marks the major syntenic breakpoint in mice and humans that defines the distal boundaries of the extended class II region in these species [26]. Two other GenScan-predicted ORFs are most likely pseudogenes as they contain exons with partial sequence similarity to DNaseII and Raftlin and transposable elements (see ORFs 4

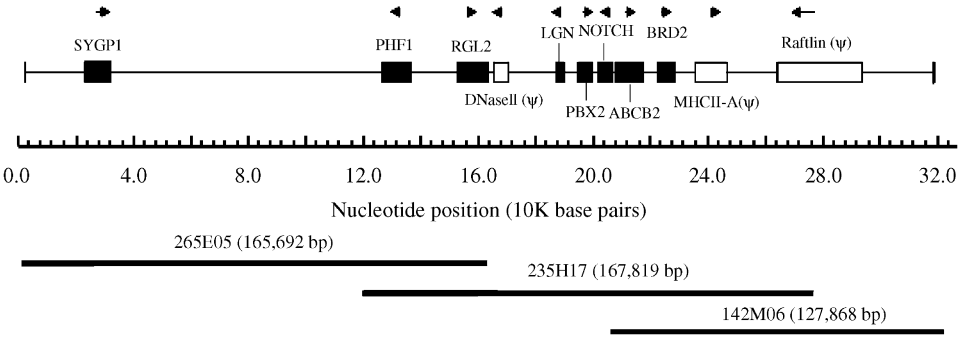

Fig. 1. Schematic map of the ABCB2 genomic region in rainbow trout. Filled and open boxes represent the expressed genes and pseudogenes, respectively. The transcriptional orientation of each ORF is shown by an arrow above each box. The three BAC clones that were used for shotgun sequence analysis are shown as thick lines with their length in parentheses.

Table 2
Putative genes identified in the ABCB2 genomic region of rainbow trout (319,300 bp). Open reading frames were identified using GeneScan and gap alignment with cDNA sequences. Putative proteins were subjected to sequence similarity searches using BLAST2 (nr database)

Gene name	Abbreviation	Alias	a.a. similarity (%)	Alignment length (a.a.)	Peptide length	Exons[a]
Synaptic Ras-GTPase-activating protein 1	SYGP1	SYNGAP	88 (*Tetraodon*)	671	1139	11
			83 (human)	673	1065	
PHD finger protein 1	PHF1	N/A	82 (*Tetraodon*)	195	295	7
			60 (human)	195	567	
Ral guanine nucleotide dissociation stimulator-like 2	RGL2	RAB2	73 (zebrafish)	386	386	17
			50 (human)	767	777	
pre-B-cell leukemia transcription factor 2	PBX2	HOX12	92 (*Tetraodon*)	312	382	8
			79 (human)	350	430	
LGN-like protein	LGN-like	N/A	60 (zebrafish)	328	328	5
G-protein signaling modulator 2	LGN	GPSM2	52 (human)	169	677	
NOTCH-like protein	N/A	N/A	70 (zebrafish)	466	507	11
Notch homolog 2	NOTCH2	hN2	61 (human)	124	2471	
Antigen peptide transporter 1	ABCB2	TAP1; RING4	99 (trout)	725	739	12
			71 (human)	575	748	
Bromodomain-containing protein 2	BRD2	RING3	69 (zebrafish)	801	814	11
			67 (human)	796	801	

[a]The number of exons in the trout orthologs for SYGP1, PHF1 and RGL2 was predicted by Genscan. PBX2, LGN, NOTCH, ABCB2 and BRD2 were predicted from gap alignment with fully sequenced cDNA clones (DR782904, DQ139863, DQ143175-9).

Table 3
Nucleotide identities between the predicted open reading frames and rainbow trout ESTs (complete cDNA sequence for ABCB2)

Gene	Genbank	Length of match (bp)	Identity (%)
RGL2	CA357310	553	97
PBX2	BX073868	584	98
	CR372297	500	97
LGN	CA376566	747	99
	CA341806	726	99
NOTCH	BX877388	774	96
ABCB2	AF115536	2485	99
BRD2	CA361769	697	100
	CA344294	689	100
	BX886126	470	94

and 11, in Fig. 1). Eleven additional Genscan-predicted ORFs were determined to be transposons and retrotransposable elements, which are a common feature of the teleost MH regions [6,12].

3.2. Expression analysis

We then assessed the expression of members of the ABCB2 locus that were not found within the trout EST gene index (www.tigr.org) to initially evaluate their function or whether they represent psuedogenes based upon their tissue-specific expression. BRD2 expression served as a positive control for our RT-PCR analysis as multiple BRD ESTs were found in the trout EST index. Primers were developed for three additional ORFs that were identified by GenScan (MHC IIA, SYPG1 and PHF1) for expression analysis within our panel of trout cDNAs from various tissues. Expression of the BRD2 homolog suggests that it may participate in the regulation of immune genes based upon its strong expression in the gut, anterior kidney and spleen (Fig. 3). No expression of the truncated class DAA ORF was detected by the RT-PCR, which coupled to the lack of ESTs for this ORF, confirms the psuedogene nature of this gene. SYPG1 was expressed in the brain, pituitary and eye suggesting that it has a similar role to the mammalian brain-specific homolog [33,34]. Finally, PHF1 had strong expression in the spleen, thereby indicating that it may be involved in regulating immune-relevant genes.

3.3. Phylogenetic and syntenic relationships

The human ABCB family encodes a broad class of genes including transporters of antigenic peptides

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Onmy-ψDAA-A2    1 --------SQRKVELGVPNTLICLVINFHPTPVHVTWTINEQPVGERT 40
Onmy-DAA-A2     1 APPHSSIYPRDDVELGVENTLICHVSAFHPAPVRVRWTRNNQNVTE-G 47
Sasa-DAA-A2     1 DPPHSSIYPRDDVDLGVENTLICHVSGFFPAPVRVRWTRNNQNLTE-G 47
Icpu-DAA-A2     1 DAPQSSIYPRDDVQLGSKNTLICHAIRFFPPPVRVRWTKNNVDVTG-E 47
Auha-DAA-A2     1 DPPEIVLFSSDKVELGVENSLICFVNHFYPPSINVTWTKNGHPVST-G 47
                            * **  *:***    * *  : * ** *   :

Onmy-ψDAA-A2   41 VSQTQYYSNEDFSFRIFSYLSITPQEGDIYSCSVGHVSLQEPFTRNW 87
Onmy-DAA-A2    48 VRLSTPYPNTDFTLNQFSSLTFTPEEGDIYGCTVEHKALTEPLTRIW 94
Sasa-DAA-A2    48 VRLSTPYPNADFTLNQFSSLPFTPEEGDIYGCTVEHKGLAEPLTRIW 94
Icpu-DAA-A2    48 SSLSQYYPNEDETFNQFSHLPFTPQEGDVYTCTVQHEALQTPDTRTW 94
Auha-DAA-A2    48 VSLSRYFPNKDQTFHQFSTLTFTPSEGDFYSCTVEHSALETPKTRIW 94
                     :  : * * :   ** *  ** *** * *:* *  *  * ** *
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Fig. 2. Amino acid alignment of an orphan class IIA exon in the trout ABCB2 genomic region with other class IIA sequences. Amino acid sequences corresponding to exon 3 of the trout DAA pseudogene were aligned (ClustalW) with rainbow trout (AJ251433), Atlantic salmon (AJ438967), channel catfish (AF103007)and cichlid (AF091557) DAA sequences. Asterisks (*) and colons (:) indicate identity and similarity respectively for all species examined. Cysteine and tryptophan residues involved in the Ig fold are in bold and the unique *N*-linked glycosylation site found in salmonids is underlined. Identical residues between the orphan class IIA exon and the expressed trout DAA exon 3 are in gray.

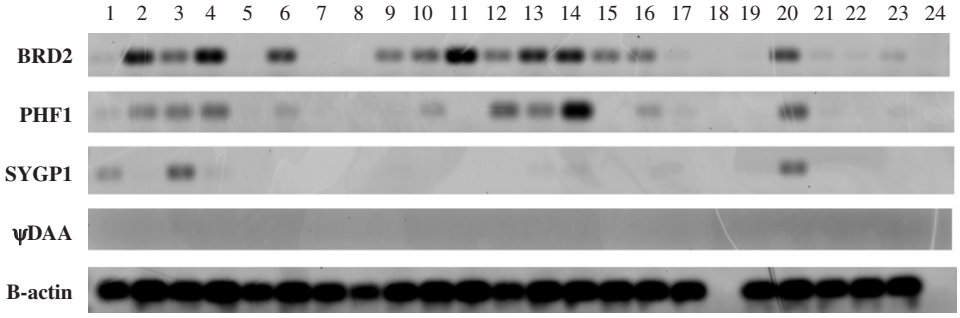

Fig. 3. Tissue-specific expression of selected genes in adult rainbow trout as measured by RT-PCR. Lane assignments: 1—brain; 2—pyloric ceca; 3—pituitary; 4—intestine; 5—heart; 6—liver; 7—ovary; 8—pancreas; 9—testis; 10—peripheral blood leukocytes; 11—anterior kidney; 12—red blood cells; 13—trunk kidney; 14—spleen; 15—stomach; 16—gill; 17—brachial arch; 18—negative control; 19—skin; 20—eye; 21—white muscle; 22—red muscle; 23—fat; 24—genomic DNA.

(ABCB2 and 3) and the multiple drug resistance (MDR) genes. ABCB3 paralogs are found within the class I regions (Chrs. 14 and 18) in trout whereas ABCB2 appears to be a single copy gene that maps to chromosome 2. Since the original description of the trout ABCB2 gene [8], an additional ABCB gene (ABCB9, aka TAPL) has been described that has features similar to both ABCB2 and 3 [42]. We therefore confirmed (Fig. 4A) that the trout ABCB2 gene is truly orthologous to human ABCB2, which is located within the human MHC class II region. The overall synteny for the trout ABCB2 chromosomal region and human chromosome 6p21 (Fig. 5) also supports that this gene is ABCB2. Finally, trout ABCB2 is expressed primarily within trout lymphoid tissues similar to mammalian ABCB2, whereas mammalian ABCB9 is expressed mainly within testis and brain [42]. Additionally, human ABCB9 is not responsive to IFNγ induction whereas trout ABCB2 is up-regulated in response to viral infection and possesses interferon regulatory sites within its promoter similar to that of human ABCB2 [43]. The latter is important for evolutionary theories of the MHC as it reveals that coordinated regulation based upon functional clustering is not required in teleosts where transcriptional regulation of the class I pathway (class IA "core", ABCB2 and TAPBP) is maintained in *cis* and *trans* likely through the downstream regulatory effects of interferons [14,43].

The four other expressed genes with high sequence similarity to human MHC homologs (BRD2, PBX2, RGL2 and SYPG1) were positioned in their respective phylogenetic trees for assessing orthology (Fig. 4). BRD2 and PBX2 belong to the four MHC paralogous groups in humans on chromosomes 1, 6 (HLA), 9 and 19 [44]. For the

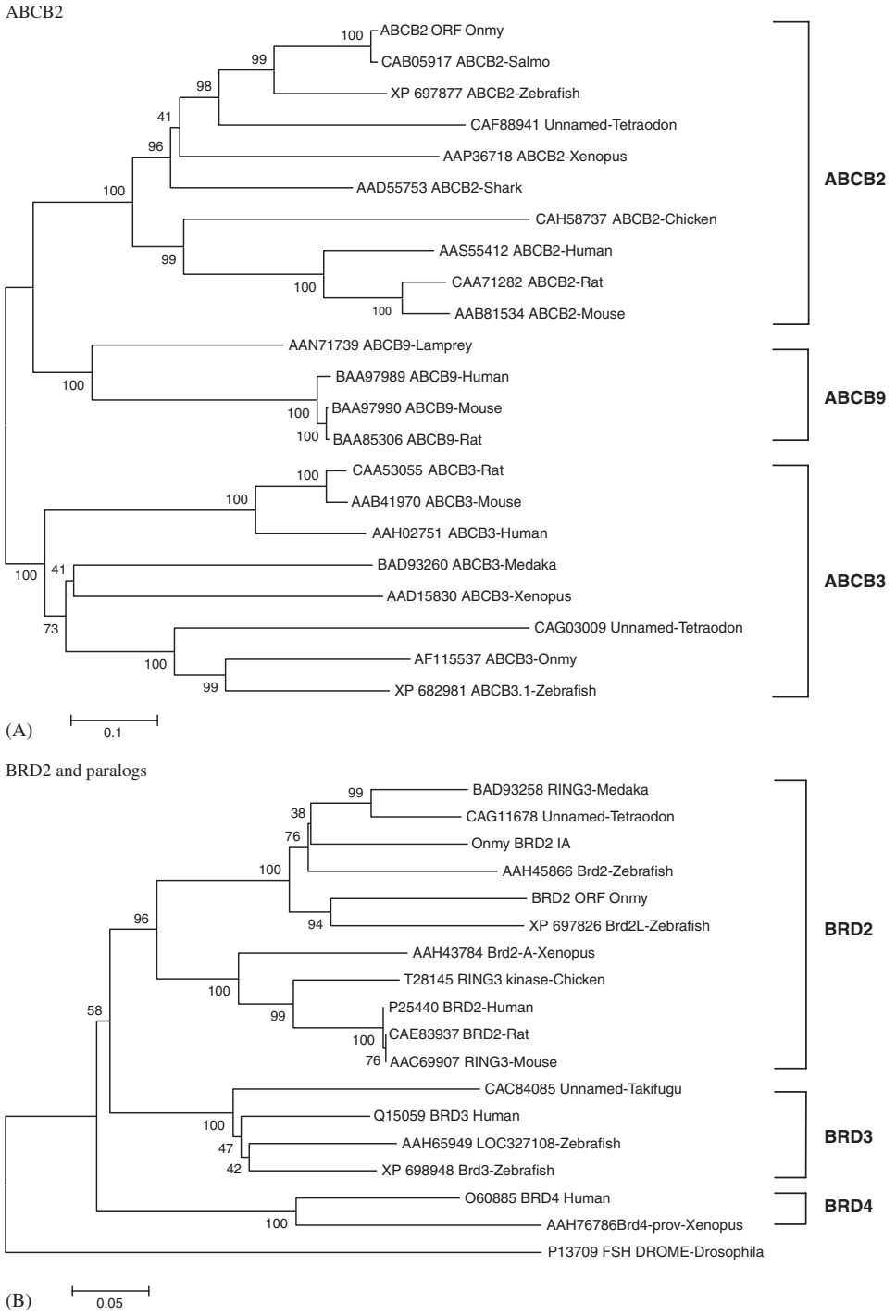

Fig. 4. (A–E) Phylogenetic analysis of the ABCB2 region. Trees (A—ABCB2, B—BRD2, C—PBX2, D—RGL2 and E—SYPG1) were generated to determine relationships between putative rainbow trout proteins (Onmy ORFs) in the ABCB2 genomic region that share sequence similarity to human and teleost proteins. Amino acid sequences were aligned (length described in Table 2) using ClustalW and phylogenetic trees were then constructed using the neighbor-joining method. Bootstrap values indicate the percentage of times that a given node could be recovered in 1000 replications and are indicated at the branch points. Each protein is indicated by the corresponding accession number, name and species.

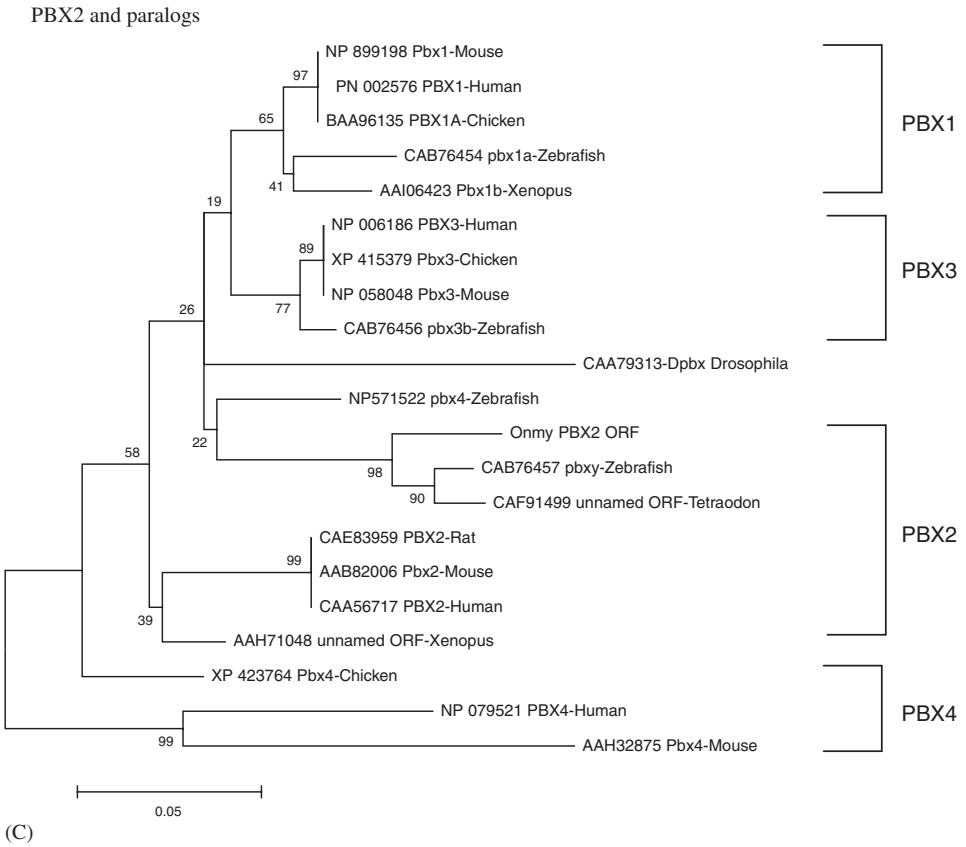

Fig. 4. (*Continued*)

BRD2 phylogenetic analysis, we included the human paralogs and sequences from zebrafish and other species that were identified by BLASTP and tBLASTn for each of the human paralogs. A clear BRD2 clade was formed (Fig. 4B); but it was divided into two sub-clades. The teleost BRD2 orthologs formed a separate sub-clade from the mammalian, chicken and *Xenopus* BRD2 orthologs. The zebrafish BRD2 (Ring3) ortholog (AAH45866) was previously mapped to chromosome 19 near the zebrafish MHC Ia core region [45]. We have identified a second BRD2 paralog within the trout MHC class IA genomic region (Hansen, manuscript in preparation), which is the homolog of zebrafish BRD2 that maps to zebrafish Chr. 19 (class IA core). The trout BRD2 paralog identified in the current study (BRD ORF *Onmy*) is the likely homolog of the zebrafish BRD2-L gene that was recently mapped to zebrafish Chr. 9. Sambrook et al. [45] recently linked ABCB2 (TAP1) and BRD2-L (XP_697826) on zebrafish Chr. 9, thus indicating conserved synteny of the two BRD2 paralogs to MHC class IA and ABCB2 regions in both zebrafish and trout. There are two zebrafish homologs for human BRD3 (human Chr. 9); Brd3-zebrafish is located on chromosome 8, while LOC327108 is on Chr. 5 suggesting the presence of BRD3 paralogs in zebrafish. The zebrafish sequence in the BRD4 cluster (Wu:fi25h02) is currently not mapped to a chromosome. For the PBX analysis, we also included the four human paralogs and their putative orthologs from other vertebrates. Trout PBX2 was tentatively positioned on the teleost node for PBX2. Zebrafish homologs were identified for PBX1, 2 and 3, while the putative PBX4 did not cluster with the other PBX4 sequences. The zebrafish PBX1a and PBX3b genes were recently mapped to chromosomes 11 and 18, respectively [45]. Finally, our phylogenetic analysis established an orthologous relationship for the vertebrate RGL2 (Fig. 4D) and SYGP1 (Fig. 4E) sequences. Although the trout and human SYPG1 genes display synteny with ABCB2 (Fig. 5), this level of synteny was not conserved with other teleosts.

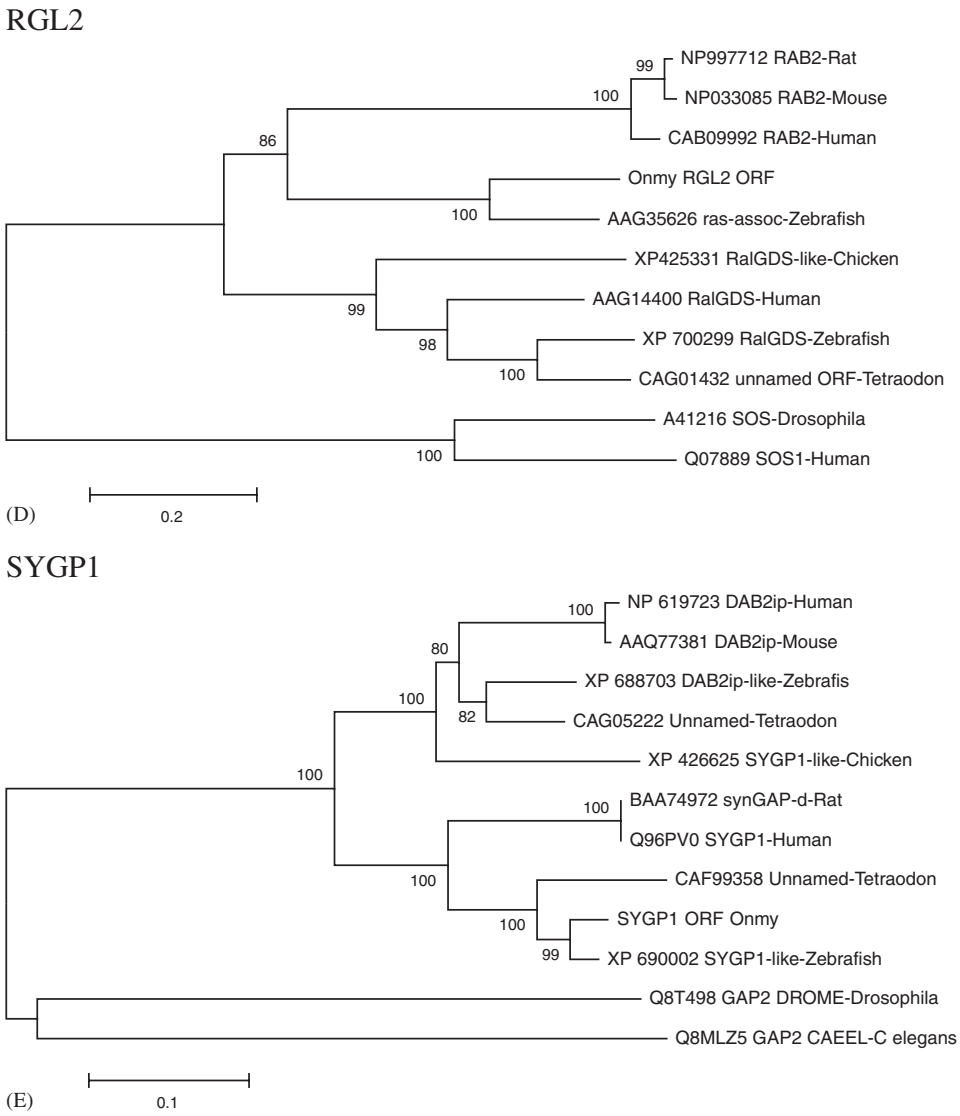

Fig. 4. (*Continued*)

We did not conduct phylogenetic analysis for the predicted trout PHF1 peptide as its length was less than 50% of the mammalian proteins. The two genes that contain LGN and NOTCH motifs share high sequence similarity with other teleosts, but not unambiguously with other vertebrates, suggesting that they are novel fish paralogs that arose from the ancient teleost genome duplication event [46]. NOTCH4 is located in the human class III region, but it appears to be absent from the zebrafish assembly or it was lost/silenced during the teleost duplication event [45]. Examination of the genomic regions surrounding Tetraodon and zebrafish ABCB2 (CAF88941 and XP_697877, respectively) revealed the presence of NOTCH-like fragments in both Tetraodon (CAF89260) and zebrafish (XP_698009). We were not able to confidently assign the NOTCH-L sequences to any of the four human paralogs as conservation varied between the analogous exons of human NOTCH1-4. The zebrafish and trout NOTCH-L amino acid sequences however displayed 70% similarity (Fig. 6) and the zebrafish ABCB2, BRD2-L and NOTCH-L sequences are linked within 50 kbp of each other on Chr. 9 (position 33 mbp), thereby further expanding the sytenic relationships for the teleost ABCB2

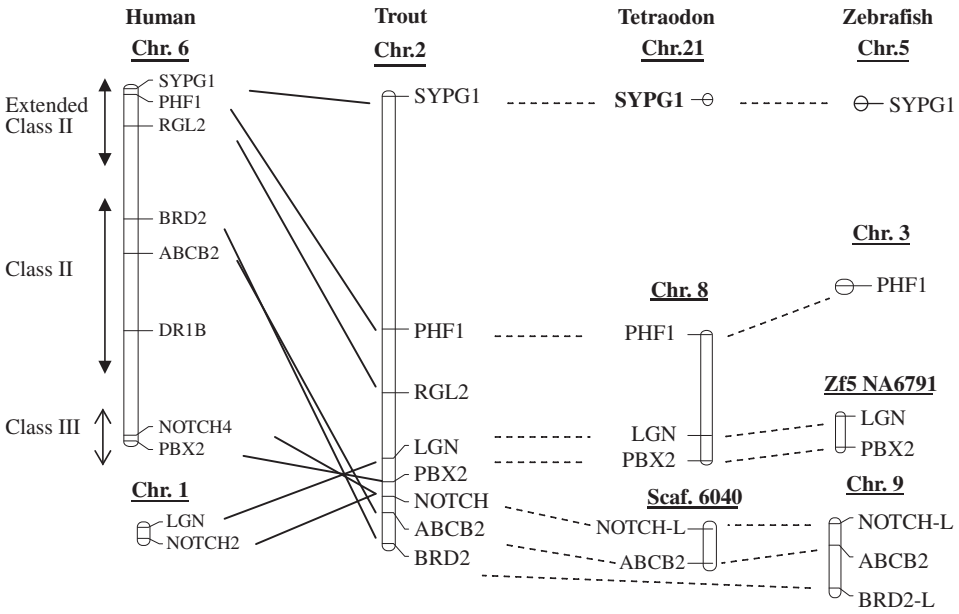

Fig. 5. Syntenic relationships of the ABCB2 genomic region. Comparative diagram of the rainbow trout ABCB2 (TAP1) region and segments of human chromosomes 6 and 1, Tetraodon chromosomes 8 and 21 and Scaffold 6040 and zebrafish chromosomes 5, 3 and 9 and Scaffold Zf5 NA6791. Orthologs were identified by phylogenetic analysis as described in the methods and in Fig. 4. Genes located on zebrafish chromosome 9 are based on Sambrook et al. [45] and BLAT analysis of the ZFV.5 assembly. Chromosomes and scaffolds are not drawn to scale. Solid lines indicate sequence similarity to human genes and broken lines indicate similarity to putative Tetraodon or zebrafish genes.

region. Although we determined that the Tetraodon BRD2 sequence (CAG11678) used in our phylogenetic analysis is part of the class I core region in this species (Tetraodon Scaf_15041), we were unable to identify an authentic Tetraodon BRD2-L paralog as found for trout and zebrafish for establishing further linkage with ABCB2.

4. Discussion

In this study, we described the genomic sequence and initial gene characterization of the ABCB2 region in teleosts. This analysis led to the discovery of conserved synteny of the trout ABCB2 region, with the human MHC class II, III and extended class II regions thereby providing a glimpse of the evolutionary history that has shaped the teleost MH regions. The average gene density for the entire contig was roughly one gene per 40 kbp; however, if the SYGP1 gene is excluded, the gene density for the segment containing genes with conserved synteny to the mammalian MHC was one gene per 15 kbp, which is similar to the gene dense nature of the human MHC [25]. Our analysis provides further evidence for the derived nature of the teleost MH regions when compared to that of the MHC architectural arrangements of sharks, *Xenopus* and mammals [13,44,47]. Recent non-mammalian genome projects have provided considerable information regarding the evolutionary history and arrangements of the MHC, where it is thought that two evolutionary mechanisms have likely accounted for the vast majority of architectural differences observed between the teleost MH loci and the "standard/ancestral" tetrapod arrangement. These principles include translocation and gene(ome) duplication followed by selective gene loss (silencing) [44,48]. For the translocation hypothesis, a small region of the MHC would be moved to a different region in the genome that would lack flanking MHC-related genes. In contrast, the basic tenet of the "duplication followed by silencing principle" is that post-duplication, genes on both duplicated regions should have remained linked with other genes (or gene fragments) found in the near vicinity of the duplication itself but that some of the linked genes have been silenced on one chromosome. Examples of putative gene silencing for the MHC have been noted in both teleosts and amphibians [49–51]. Therefore, one of the aims of

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ZF-XP_698009    1 SPN-EPYSSCPAGKSCQARFGNGVCDQDCSTSDCLRDGFDCLKDKGTCVP  49
RT-DQ143177     1 SPGGDPWAQCPS-RQCKATFGDGSCDKECTEPECLRDGFDCLRDKGHCNS  49
                  **: :* : **: : *:* ** * **::*:  :*********:*** *

ZF-XP_698009   50 GHIQYCRNHYDNGHCDQGCNNAACGWDGSDCHRNHYPIWAKGSLILHTRI  99
RT-DQ143177    50 GHIHYCRDHYANSYCDQGCESAACGWDGSDCHRHHSPLWAKGTLLLHTHV  99
                  ***:*** ** *  *****  ************:* *:****:*:***::

ZF-XP_698009  100 PFLKGQIQNSSLLWPLSTVLQTSVKLRGTAPLQTSTDLNTVDTRQLAEMY 149
RT-DQ143177   100 PLQQGTFSNSSLLWALSTLLQTPLKLRGMVPLDPSKDLFTFNPQQLENLL 149
                  *  :*   ****** ***:*** :****  **  * ** *   :**  :

ZF-XP_698009  150 THTLASDSDGSVLFLQLDNRPCSRLLSTCFDYAAEAAHFLQAVLTQNQPS 199
RT-DQ143177   150 AQASSDDSNGSLLFLQVDNRPCSHLPSTCFPYAIEAANFLRAATSSTRVS 199
                  ::: : ** **:****:******:* **** ** ***:**:*  : :: *

ZF-XP_698009  200 FPMIPEI-QAAIISVRGVDEEIGARDE-PKLPKEERNARD--SPVWMWPV 245
RT-DQ143177   200 VPSHPELK--AIISVRGVGEEIGGREEDPVEEKEETNGGTGATPPWLWAV 247
                   *  **:   ******** **** *:* *   *** *     :* *:* *

ZF-XP_698009  246 VGVATGLCVALAVIITVLLLWFKRRRARRDGADRLRHRSTLTDSN---NA 292
RT-DQ143177   248 IGVATGLVLALVLMVVLAIRRVRRRRAEREGGERVRHRSTVTENDSGANV 297
                  :****** :** ::: : :   :**** *:* :*:*****:*:     *

ZF-XP_698009  293 SKAWTRHSEEK--RSRSGREKDRNGMKKNKKGKES-GKRRKEPLGEDAIR 339
RT-DQ143177   298 AKAWAQHTPHREQRGRTGREKDRNGIKK-KKAKEAEKKRRRDPLGEDAIR 346
                  :***::*:  :  * *:********:** ** **:  ***::********

ZF-XP_698009  340 MRPLRKELDIGSDTDVTQSSMEDINKSICDHRSPEQKHY----SHQQP-- 383
RT-DQ143177   347 LRPLKKDLDIGSDTDFTQSSMEDINRSICDHRTQEQKHYRSPPSHPQPPI 396
                  :***:*:******** *********:******: *****    ** **

ZF-XP_698009  384 --RMLAPPRGWERNAVPAPQ-RPPRTAAPVQWCGPDGSVVLIRAVRSGLD 430
RT-DQ143177   397 QPPLLAPPRGWERNAVPSTQHRAPNQSGSVQWCGPDGSVVLIRAVRSGLD 446
                     :*************: * * *  :  ********************

ZF-XP_698009  431 RVVLELLRAGVPVNNTDHTGKSTIHPSLTSSFLQLG 466
RT-DQ143177   447 RVVLELLRAGVPVNNTDHTAP-LLPPSQTDTEVNVP 481
                  ******************    : ** * : :::
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Fig. 6. Partial alignment of zebrafish and rainbow trout NOTCH-like peptides that are linked to ABCB2. The zebrafish sequence (ZF, XP_698009/692917) was aligned with DQ143177 (trout-RT) using ClustalX. Asterisks (*) and colons (:) represent identical and similar amino acids, respectively.

this study was to assess the evolutionary history of the teleost ABCB2 region as the genes encoded within this region are typically found embedded within the class II and extended class II regions in most gnathostomes.

A few years ago, Abi-Rached et al. [52] described the likely organization of the ancestral MHC using amphioxus to assess the evolutionary events (en bloc duplications) that have led to the generation of the four MHC paralogous regions in mammals. A series of ''MHC anchor genes'' were used to show that an initial large-scale duplication event occurred after the divergence of the cephalochordates and gnathostomes. At some point prior to the emergence of the gnathostomes, it is thought that class I and II gene became linked with the proto-MHC [44,52]. Therefore, the characterization of conserved regions of the MHC between distant genomes represents a critical method for tracing the evolutionary history of MHC arrangements during the speciation of gnathostomes. The separation of the MHC into the class I and II regions is a prominent feature of the teleost MHC arrangements such that they are now commonly referred to as MH regions as opposed to a true complex. As the genes encoded within these divided ''MH regions'' are all present within a single cluster in the most primitive gnathastomes [53,54], the extreme differences in the genomic architecture of the bony fish MHC is most likely due to a combination of lineage-specific events including en bloc gene(ome) duplication, inversions, deletions, insertions and translocations.

Most comparative biologists accept that a genome-wide duplication event (325–350 Mya) occurred in the teleost lineage after it split (Fig. 7) from the lobe-finned lineage (which includes the

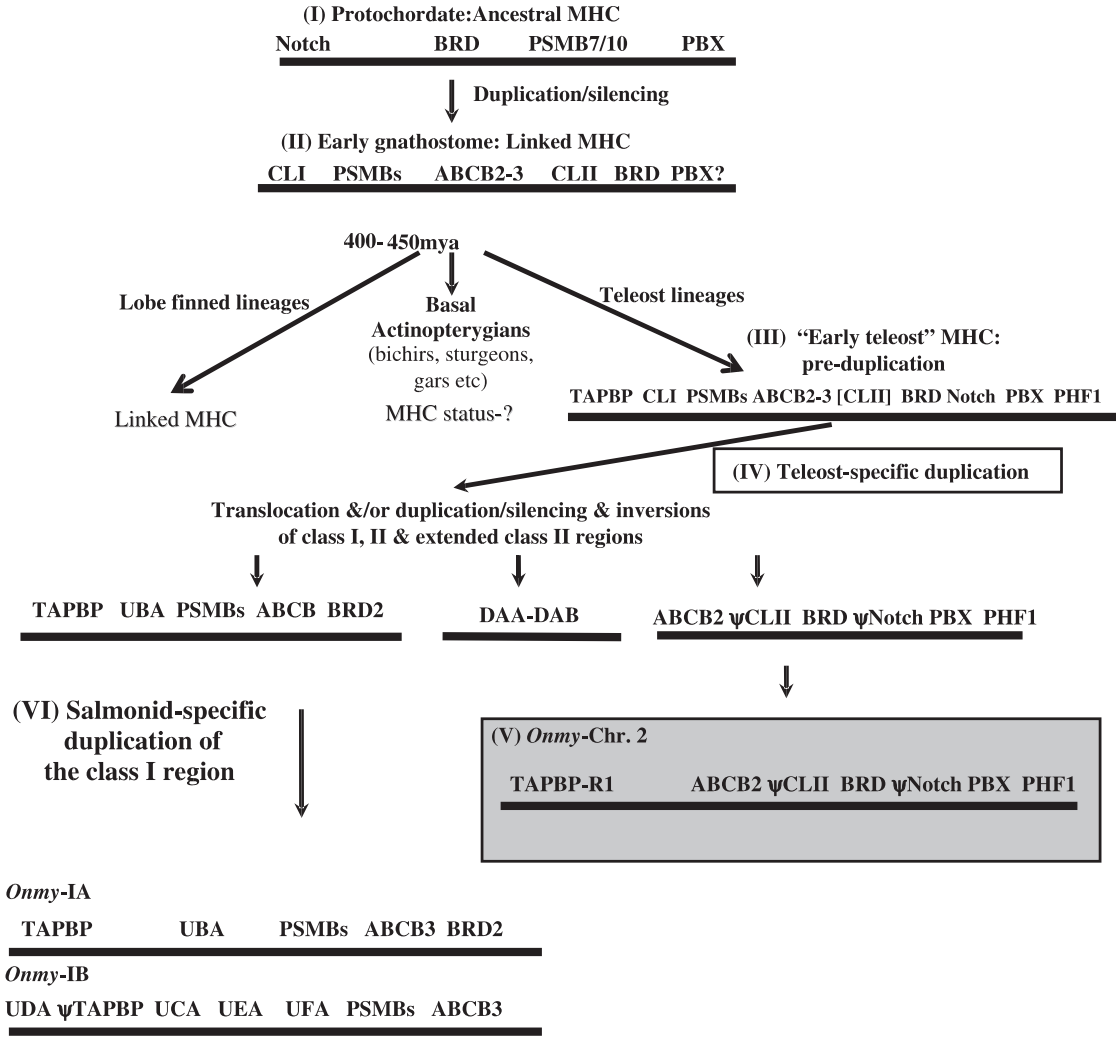

Fig. 7. Proposed evolutionary scenario giving rise to the ABCB2 region in teleosts. The ancestral/protypical MHC (I) described by Abi-Rached et al. [52] is shown indicating the arrangement of critical anchor genes prior to the radiation of gnathostomes. The next cluster of genes (II) indicates the expected linked nature of the MHC in an early gnathostome (based upon the MHC of elasmobranches, BRD linkage—Martin Flajnik, personal communication) prior to the divergence of lobe-finned, basal teleosts and the true teleost lineages, which occurred 400–450 Mya [46,59]. The next grouping (III) represents the likely MHC arrangement in an ancestral teleost where upon the teleost duplication/silencing event (325–350 Mya) generated the ABCB2 region (IV) and possibly the DAA–DAB region. Alternatively, the DAA–DAB region of teleosts was generated by translocation as suggested by Kuroda et al. [48]. Cluster (V) represents the extended organization of the trout ABCB2 region including the linkage of TAPBP-R. Finally, (VI) depicts the salmonid-specific duplication of the class I region which occurred approximately 60 Mya according to Shiina et al. [12].

lineage leading to tetrapods) 400–450 million years ago, and that only a subset of these duplicated genes have been retained in the genomes of modern teleosts [46,55–57]. Hoegg et al. [58] have recently shown evidence that the teleost duplication event occurred after the split between the "basal/non-teleost" fish and the true teleosts. The date of the divergence of teleosts from the basal fish and lobe-finned lineages has allowed for a significant time-frame for the duplicated genes to be sorted out via neo/subfunctionalization, divergent resolution and simple gene loss [57,59]. This has been exemplified in the recent analysis of the zebrafish and Tetraodon genomes [59,60], where in depth EST and gene map analysis demonstrated that ~25–30% of the zebrafish and Tetraodon genes remain as duplicates. One important aspect of these studies was that the different duplicates had different evolutionary histories in these two teleosts as they were found within different regions of these genomes and that

different duplicated genes have been lost in these two teleosts even though the percentage of duplicates has been retained [59]. These studies also showed a tendency for genes on one chromosome to have duplicate sets on a single, separate chromosome as would be anticipated by a single whole or partial duplication event during teleost evolution.

Our working theory for the ABCB2 region is that the ABCB2 and 3 genes were separated during the teleost duplication event, which integrated portions of the primitive class II region including the duplication of BRD2 (Fig. 7). The relative synteny of the genes in this region among teleosts and the lack of their presence in the class I region of teleosts (aside from BRD2) strongly suggests that this region was generated during the teleost duplication event. The fact that the BRD paralogs are linked to both the teleost ABCB2 and class I region (near ABCB3) further suggests that the ABCB2 region was derived via gene duplication and not translocation. The identification of PBX and BRD orthologs and paralogs in teleost fish is important as it sets the stage for examining the generation of the four MHC paralogous regions that are found on human chromosomes 1,6, 9 and 19. The four human parologous regions are thought to be derived from en bloc or whole chromosomal duplication events that occurred in a common ancestor of both agnathans and gnathostomes and subsequently again during the speciation of gnathostomes [13,44,52]. As mentioned earlier, BRD and PBX along with NOTCH-like genes comprise anchor genes of the ancestral proto-MHC that has been described for amphioxus [52] and thus their identification serve as useful markers for the future assessment of MHC paralogous regions within teleosts. We recently reported our characterization of TAPBP and TAPBP-R paralogs in trout [14], where we showed that TAPBP-R is tightly linked to the ABCB2 region on chromosome 2 in trout. In addition, BLAT analysis demonstrates that the Tetraodon homolog for TAPBP-R maps to chromosome 8, which also houses PHF1 and PBX2 (Fig. 5) and likely ABCB2/Notch (unassigned region) suggesting that this linkage was formed in a common ancestor during the teleost duplication event. Along the same lines, phylogenetic analysis of BRD2 in teleosts indicates that the generation of the BRD2 paralogs in trout and zebrafish occurred within a common ancestral teleost during the teleost-specific duplication event as the branch lengths and symmetrical topologies are consistent with a single regional duplication event (en bloc). Interestingly, the apparent tissue-specific expression of the BRD2 paralog that is linked to ABCB2 (Fig. 3), is different from its paralog, which is found in the class IA region and displays ubiquitous expression (data not shown). The tissue-specific expression of the ABCB2-linked BRD2 paralog may be the result of regulatory divergence post duplication.

In zebrafish the class I and II "core" regions are found on chromosomes 19 and 8, respectively. Although there are several class IIA and B genes in zebrafish that are scattered on different chromosomes, only two tightly linked, functional DAA and DAB genes are known to be expressed. Not surprisingly though, Sambrook et al. [45] recently noted the presence of class IIA and B fragments that are approximately 22 mbp telomeric to the class I core region providing evidence for the ancestral linkage of functional class I and II loci. Genomic examination of the class II region in zebrafish failed to identify additional MHC-related genes within a 1 mbp region flanking the functional DAA–DAB genes supporting the theory [48] that the separation of the functional class I and II loci was likely due to a single translocation event. Alternatively, given the presence of duplicated class II genes in teleosts, this region could have been generated via a duplication event followed by translocation. Owing to the fact this arrangement is found in all teleosts studied to date, this particular event likely occurred within an ancestral teleost prior to/during the teleost-wide duplication event. During our analysis of the ABCB2 region, we identified a single DAA pseudogene. The functional DAA–DAB locus is found on chromosome 14 in rainbow trout. Previously, Ristow et al. [61] identified a second potential genomic copy of the MHC class IIB chain in rainbow trout that was not linked to DAA–DAB (Chr. 14) based upon Southern blotting, but all evidence to date indicates a single expressed DAA–DAB locus in salmonids [27,62]. The traces of a second genomic copy of DAA in the ABCB2 region might suggest that an additional DAB copy could be located in this region based upon the tight linkage of DAA and DAB in salmonids [3,4]. However, given that multiple copies of class IIA and B-like genes are found scattered throughout the zebrafish genome [5,45] thereby representing remnants of the teleost duplication event, the true significance of the trout DAA pseudogene in the ABCB2 region is presently unclear.

In zebrafish, medaka, fugu and Tetraodon the class I core region is found as a single entity, but in salmonid fish the class I core region has undergone an intrachromosomal duplication event such that two bone fide class I regions are found where each region contains MHC class I, proteosome subunits, antigen transporters (ABCB3) and tapasin genes [4,12,14]. The results for the salmonid class I regions (IA and IB) and the ABCB2 region are consistent with a duplication event followed by selective silencing (i.e., deletion or gene inactivation) of neighboring MHC loci; however the timing of these two events is greatly different. The ABCB2 region was most likely derived during the teleost-specific duplication whereas the generation of the duplicated salmonid class I region corresponds to the proposed salmonid-specific genome duplication event that occurred prior to the speciation of the salmonids. Along this line, recent analysis of one of our rainbow trout BAC libraries [15] suggested that 60–70% of the loci are duplicated based upon contig analysis which when compared to the findings for zebrafish and Tetraodon (approximately 30% of the genes are duplicated), reinforces the theory of the salmonid-specific duplication. Furthermore, Shiina et al. [12] estimated that the salmonid class I duplication event occurred around 60 Mya based upon comprehensive sequence analysis of the duplicated class I regions which is in full agreement with the genome-wide duplication time frame (25–100 Mya) proposed by Allendorf and Thorgaard [63].

4.1. In summary

The arrangement and close proximity of genes in the ABCB2 region that are syntenic to the human MHC are likely due to a combination of events involving duplication, gene silencing, inversion and likely translocation as suggested for portions of the teleost and *Xenopus* MHC [45,47,48]. Based upon this study, it appears that trout provide a unique model for assessing the functional significance, if any, of conserved synteny for the ABCB2 region in bony fish with that of the mammalian MHC owing to the conserved synteny within this region between these two gnathostomes. However, syntenic comparison with other teleosts was limited, as most of these genes have not been assigned to specific chromosomes and linkage groups in Tetraodon and zebrafish. In-depth syntenic comparisons are complicated in the teleost genomes because of the fragmented genomic organization of the teleost MHC loci and the presence of additional paralogs [45] and thus will not likely be resolved until these genomes are fully annotated. Conserved physical linkage of genes across species such as the linkage of MHC class I antigen processing and presentation pathway is thought to be the result of functional co-evolution leading to improved fitness. Whether this holds true for the multiple MH regions in teleosts awaits further analysis.

Acknowledgments

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